

# Characterization of a Binding Protein for Leukemia Inhibitory Factor Localized in Extracellular Matrix

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**Abstract.** Leukemia Inhibitory Factor (LIF) interacts with two classes of high affinity binding sites on rat UMR cells cultured in monolayer. One class of binding sites was found to be localized in the extracellular matrix (ECM) after removal of cells from the culture dish. The interaction of LIF with ECM-localized binding sites is not dependent upon either glycosylation of LIF or the presence of extracellular glycosaminoglycans. Chemical cross-linking studies demonstrate that LIF interacts with a 200-kD cell-associated protein and a 140-kD ECM-localized protein. A 140-kD pro-

tein could also be specifically precipitated from solubilised metabolically radiolabeled UMR ECM by antibodies directed against LIF by virtue of its ability to form a stable complex with unlabeled LIF. In addition, soluble LIF associated with this ECM-localized protein is biologically active in terms of inhibition of ES cell differentiation. The properties of ECM-localized 140-kD species are very similar to those of the secreted form of the LIF receptor suggesting that the ECM localization of LIF and LIF signal transduction may be closely coupled.

**L**EUKEMIA inhibitory factor (LIF)<sup>1</sup> is a polypeptide regulatory factor which exhibits a broad range of biological activities on a wide variety of cell types in vitro. These include pluripotential embryonic stem cells (Smith et al., 1988), primordial germ cells (Matsui et al., 1992), myeloid cell lines (Moreau et al., 1988; Hilton et al., 1988), adipocytes (Mori et al., 1989), hepatocytes (Baumann and Wong, 1989), and peripheral neurons (Yamamori et al., 1989). Chronic administration of LIF to adult mice results in a complex syndrome which includes wasting, behavioral abnormalities and alterations in bone deposition (Metcalf et al., 1989). The generation of homozygous LIF<sup>-</sup>/LIF<sup>-</sup> mice has also indicated that LIF is involved in the maternal control of embryonic implantation (Stewart et al., 1992). These broad ranging biological actions have led to the proposal (Metcalf, 1991; Heath, 1990) that the action of LIF in vivo is confined, by some means, to a strictly local mode of action within tissues.

Analysis of LIF gene expression has revealed two forms of LIF transcript which differ at their 5' end as a result of the use of alternative transcriptional start sites (Rathjen et al., 1990a). These two LIF transcripts are predicted to encode LIF proteins which differ at their amino terminus. A variety of evidence indicates that the two forms of LIF protein produced from these alternative transcripts exhibit distinct cellular locations and biological activities. First, analysis of LIF gene expression has revealed that the two transcripts are

differentially expressed in many cell types both in vitro and in vivo (Rathjen et al., 1990b) suggestive of a specific role for each form of the protein. Second, cells which express LIF transcripts initiated from the distal transcriptional start site (D-LIF) release LIF protein into the culture media whereas cells which express transcripts initiated from the proximal start site (M-LIF) express a form of LIF which is tightly associated with the extracellular matrix (ECM) and not released into the culture media. Finally, the association of LIF with the ECM has important consequences for the action of LIF in vivo since Conquet et al. (1992) have shown that the expression of M-LIF cDNAs in the preimplantation embryo results in arrest of gastrulation, whereas expression of D-LIF cDNA has little phenotypic effect.

While it seems clear that the association of LIF with the ECM has important biological consequences, the molecular mechanisms underlying this phenomenon remain to be defined. Here we report that the association of LIF with the ECM of cells cultured in vitro is mediated by a matrix-localized LIF binding protein whose biochemical properties resemble those of the soluble form of a transmembrane LIF receptor. This finding suggests that there may exist an intimate relationship between the mechanism of LIF presentation to cells and the mechanism of target cell response.

## Materials and Methods

### Cell Culture

UMR 106-06 cells (Allan et al., 1990) were maintained in DME F12 supplemented with 10% FBS, 2 g/liter Na<sub>2</sub>CO<sub>3</sub> (all from Flow Laboratories, Irvine, Ayrshire, Scotland), 2 mM glutamine, 50 µg/ml streptomycin and

1. *Abbreviations used in this paper:* ECM, extracellular matrix, ES, embryonic stem; FN III, type III fibronectin; GAG, glycosaminoglycans; LIF, leukemia inhibitory factor.

50 IU/ml penicillin (all from Sigma Immunochemicals, St. Louis, MO). UMR 106-06 cells were responsive to LIF as determined by an inhibitory effect of LIF on their proliferation (data not shown).

The embryonic stem cell (ES) cell line, E14 wild type, was cultured in DME F12 medium supplemented with 15% FBS, 2 g/l Na<sub>2</sub>CO<sub>3</sub>, 2-mercaptoethanol (0.14 mM), 2 mM glutamine, 50 µg/ml streptomycin, 50 IU/ml penicillin and 10 ng/ml of recombinant LIF prepared as described below.

Cell-free ECM was prepared by detachment of cells with 0.02% EDTA in PBS (Rheinwald and Green, 1975). Extraction of matrix proteins was achieved by incubation of the cell-free matrix for 6 h at 4°C in a guanidium buffer (4 M guanidium-Cl, 50 mM Na-acetate, pH 6, 2% Triton X-100, 1 mM PMSF, 1 µg/ml aprotinin), as described by Saksela et al. (1990).

### Recombinant LIF Preparation and Iodination

Recombinant LIF (rLIF) was prepared from the prokaryotic expression vector system p<sub>gex</sub>-2T (Pharmacia Fine Chemicals, Piscataway, NJ). Murine LIF cDNA encoding for the diffusible form excluding the signal sequence, was cloned into the p<sub>gex</sub>-2T plasmid at the BamHI site of the polylinker. This allowed for inducible expression of a glutathione-S-transferase-LIF fusion protein (gex-LIF), whereby the LIF protein is fused to the COOH-terminus of gex protein from *Schistosoma japonicum*. Intracellular fusion protein (gex-LIF) was recovered from *E. coli* lysates by affinity chromatography using a glutathione Sepharose gel matrix (Smith and Johnson, 1988). Pure recombinant LIF was isolated from this system by cleavage of the fusion protein by thrombin at a unique site introduced between the glutathione and LIF protein junction. This LIF was further purified by reverse phase HPLC using a reverse phase C8 column (Ultrapore RPMC; Beckman Instruments, Inc., Fullerton, CA). The column was equilibrated with 0.1% TFA/20% Acetonitrile and acidified samples were applied at a flow rate of 1 ml/min. After washing the column with 0.1% TFA/20% acetonitrile, murine LIF was eluted with a linear gradient of 20–70% acetonitrile, at a 1% incremental increase of acetonitrile per min for 60 min. Murine LIF eluted from the column at a nominal gradient of 0.1% TFA/47% acetonitrile. This material was purified to homogeneity as determined by SDS-PAGE, with a relative molecular mass of 20 kD. Biological activity of the purified protein was assessed by its inhibitory effect on ES differentiation (Smith et al., 1988) and its proliferative effect on DA-la cells (Moreau et al., 1988), its specific activity in the latter was determined to be 1–4 × 10<sup>6</sup> U/mg (whereby one unit of LIF activity is defined as the amount required to induce a half-maximal proliferative response of DA-la cells).

Purified recombinant LIF was iodinated using the Iodo-gen method (Fraker and Speck, 1978). LIF (3 µg) in 0.1 M phosphate buffer, pH 7, containing 1% PEG was added to a tube precoated with 1 µg Iodo-gen (Pierce, Rockford, IL). Carrier-free [<sup>125</sup>I] (1 mCi; A.S.:15 mCi/µg, Amersham International, Buckinghamshire, England) was added to the mixture and incubated 20 min at room temperature. The reaction was terminated by removing the solution from the tube and renaturation of the protein was achieved by addition of DTT to a final concentration of 12.5 mM. [<sup>125</sup>I]-LIF was separated from unbound [<sup>125</sup>I] by gel filtration through a Sephadex G-25 column (PD-10, Pharmacia Fine Chemicals) pre-equilibrated with PBS containing 0.1% BSA. Fractions were monitored for radioactivity and those eluting in the void volume were pooled and stored at 4°C. Preparation of [<sup>125</sup>I]-LIF used for experiments in this paper had specific activity in the range of 2–4 × 10<sup>18</sup> dpm/mol. Radiolabeled [<sup>125</sup>I]-LIF was determined to be biologically active as measured by differentiation inhibition assays on Embryonic Stem (ES) cells (Smith et al., 1988).

### Polyclonal Antibodies

Gex-LIF fusion protein was used to inoculate rabbits for the generation of polyclonal anti-LIF antibodies. Bound gex-LIF fusion protein (as described above) was released from the glutathione Sepharose matrix by direct exchange with reduced glutathione (1 mM) and then further purified by electroelution from an SDS-polyacrylamide gel. Specific recognition of antiserum to the LIF protein was verified by Western blot analysis and immunoprecipitation experiments (results not shown) whereby anti-LIF serum immunoprecipitated a 43-kD species in the supernatant of cos cells transfected with the LIF gene that was not resolved by pre-immune serum.

Anti-fibronectin, anti-transferrin receptor, and anti-vinculin antibodies used to perform Western blot analyses were all obtained from Sigma Immunochemicals.

### Radioligand Binding Analysis

Binding assays were performed on confluent adherent monolayers of UMR

cells grown in 5-cm-diam dishes (5 × 10<sup>6</sup> cells per dish) in 2 ml of binding buffer (DME containing 25 mM Hepes, pH 7.4, 0.1% BSA, 0.1 mM PMSF, and 1 µg/ml each of aprotinin, leupeptin, and pepstatin). Cells were incubated with increasing amounts of radiolabeled LIF with or without a 500 fold excess of unlabeled LIF, in duplicate for 3 h at 4°C. Cells were then washed twice with cold binding buffer and once with binding buffer containing 0.2 M NaCl. Cells were removed from the dishes with a lysis buffer (20 mM Hepes, pH 7.4, 0.15 M NaCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 1% Triton X-100) and cell-bound radioactivity was determined by measurement in a gamma counter (LKB Instruments Inc., Newbury Park, CA). Specific binding was calculated by subtracting radioactivity bound in the presence of excess unlabeled LIF from total binding. Generally, nonspecific binding represented 25–30% of maximal binding. Variation between replicated determinations was <10%. For determination of binding constants, saturation binding data were analyzed using the LIGAND fitting program (Munson et al., 1980).

To achieve chemical cross-linking of proteins on cells, cells were washed once with binding buffer without BSA after the binding reaction. The cross-linking reaction was initiated by addition of *N*-hydroxy-sulfosuccinimide (Sulpho-NHS; Pierce) (5 mM) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, Pierce) (20 mM). The reaction was performed in binding buffer (without BSA) during a 30-min incubation at 4°C and then quenched by addition of 10 mM tris-Cl, pH 7.4, containing 10 mM glycine. After extensive washing with cold binding buffer, cells were solubilized with 0.1 ml SDS sample buffer (Laemmli, 1970), sonicated for 30 s to reduce viscosity, and analyzed by SDS-PAGE.

### Cell Labeling, Lysis, and Immunoprecipitation

For metabolic labeling with [<sup>35</sup>S]methionine, subconfluent cells grown in 10-cm-diam dishes were washed twice with PBS, and then serum-starved in 2 ml methionine-free modified MEM, supplemented with 5% dialyzed FBS (GIBCO BRL, Gaithersburg, MD) for 30 min at 37°C. [<sup>35</sup>S]methionine (90 µCi/ml; A.S.:1,000 Ci/mmol; Amersham) was then added and cells were metabolically labeled overnight. After labeling, cell supernatants were collected and clarified by centrifugation for 30 min at 4°C.

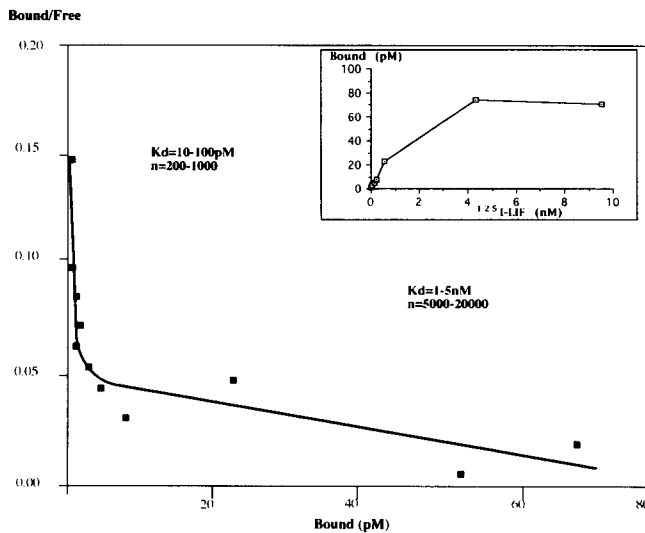
For immunoprecipitation of matricial proteins from [<sup>35</sup>S] radiolabeled cell-free ECM, guanidium was first exchanged for 8 M urea by gel filtration through a Sepharose G-25 column that had been pre-equilibrated with 8 M urea in 50 mM Na-acetate buffer, pH 6, containing 0.5% Triton X-100, 0.15 M NaCl, 1 mM PMSF, 1 µg/ml each of aprotinin, leupeptin and pepstatin, and then eluted with the same buffer. The eluted radiolabeled material was pooled and the sample was adjusted with 50 mM Na-acetate, pH 6, to a final concentration of 2 M urea.

Before immunoprecipitation with polyclonal antibodies to LIF, clarified supernatant and matrix extract were precleared by incubation with protein A-Sepharose (100 µl of 10% suspension [vol/vol]; CL4B; Pharmacia Fine Chemicals) for 30 min at 4°C. Supernatants were transferred to new tubes containing crude anti-LIF antiserum at 1:100 final dilution and incubated for 2 h at 4°C. As controls, equivalent amounts of pre-immune serum were used. Protein A-Sepharose (100 µl of 10% suspension) was then added. After a further 2-h incubation at 4°C, immune complexes were centrifuged through a sucrose gradient (10–20%), washed twice with 0.1 M NaCl, 10 mM tris-Cl, pH 8, 0.1% NP-40, 0.1% SDS, resuspended in SDS-sample buffer, heated to 100°C for 3 min, and then electrophoretically resolved through polyacrylamide gels containing SDS.

## Results

### Characterization and Localization of LIF Binding Sites

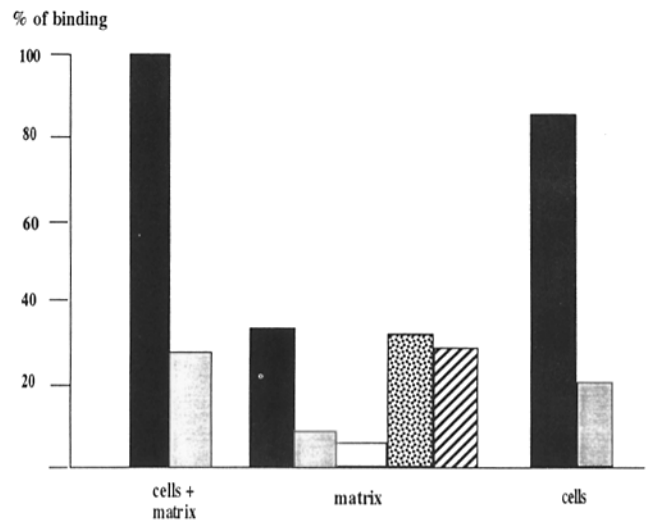
The purification and amino acid sequencing of LIF from the ECM of cells which express predominantly M-form transcripts has revealed that the protein exists in the matrix in its mature form lacking the secretory signal sequence (Rathjen et al., 1990a). This finding suggested that the association of LIF protein with the ECM is an inherent property of the mature form of LIF protein. Thus, we accordingly examined the ability of exogenous LIF to associate with the ECM of cultured cells. For the purposes of these experiments we used, as a ligand, recombinant nonglycosylated murine LIF



**Figure 1.** High and low affinity binding sites on UMR 106-06 cells. Scatchard analysis of one of the three saturation experiments performed on cells grown on their matrix. (*Inset*) Saturation curve for specific binding ( $n = 3$ ).

produced in bacteria which corresponds in sequence to the mature form of the protein with the addition of two amino acid residues at the  $\text{NH}_2$ -terminus and as a target cell, rat UMR osteosarcoma cells. UMR cells have been reported to express relatively high numbers of LIF receptors but have low levels of endogenous LIF expression (Allan et al., 1990).

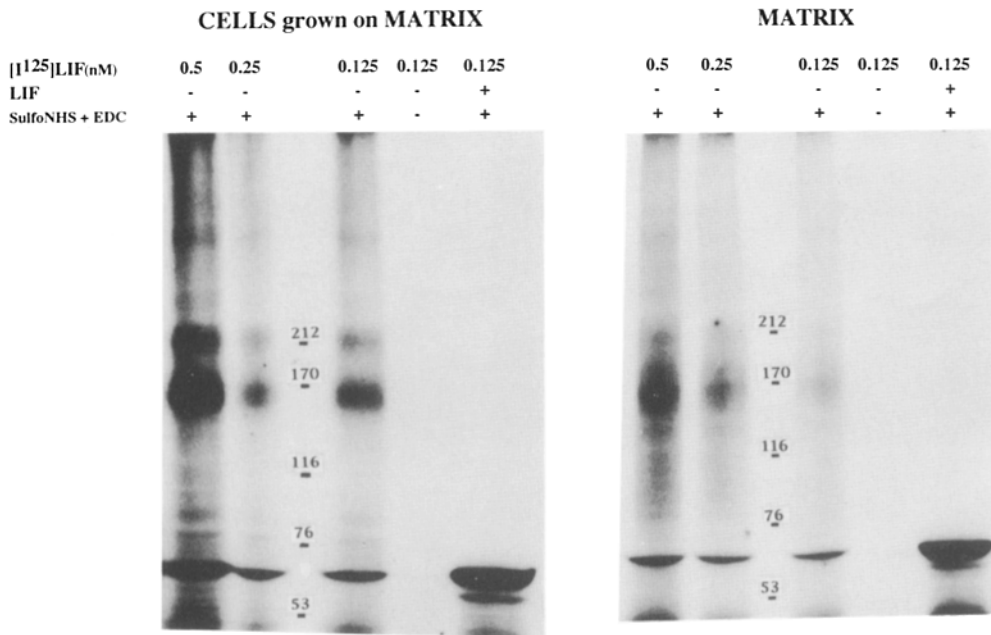
The expression of specific LIF binding sites on UMR cells grown in monolayer was examined by radioligand binding assay using  $^{125}\text{I}$ -labeled LIF. A scatchard transformation of the binding isotherm of LIF to UMR cells, applying the LIGAND curve fitting program (Munson et al., 1980; Draper and Smith, 1966), revealed two classes of binding sites (Fig. 1): a majority class (apparent  $K_d = 1-5$  nM, 5,000-20,000 sites/cell) and a minority class (apparent  $K_d = 10-100$  pM, 200-1,000 sites/cell). These findings are in accord with previous studies of LIF binding sites expressed by UMR and other cells (Hilton et al., 1988, 1991; Godard et al., 1992) but do not distinguish between LIF binding sites associated with cells or associated with the ECM. Cell-free ECM preparations of UMR cells were derived from monolayer cultures in which the cells had been removed after dispersal with EDTA. Immunochemical analysis of the residual ECM associated with the culture dish indicated that it was significantly depleted in marker proteins corresponding to either cell membranes (transferrin receptor) or cytoskeleton (vinculin) but did contain the ECM located protein fibronectin (data not shown). A comparison of the specific binding of  $^{125}\text{I}$ -LIF to either intact cell monolayers or to the cell-free ECM preparation revealed that  $\sim 35\%$  of the total specific binding was derived from binding to the ECM (Fig. 2). These extracellular LIF binding sites were derived from UMR cells since the specific binding to virgin tissue culture plastic was insignificant. Since it is well established that other growth factors may associate with the ECM by means of association with the extracellular carbohydrate side chains of glycosaminoglycans (GAGs) (Vlodavsky et al., 1987; Andres et al., 1990) we examined the effect of treating the



**Figure 2.** Specific binding of  $^{125}\text{I}$ -LIF to ECM is not abolished by digestion of glycosaminoglycans. Binding of  $^{125}\text{I}$ -LIF (5 nM) is expressed as the percentage of the total amount of radioactivity bound to cells grown on their matrix, both total ( $\blacksquare$ ) and nonspecific ( $\square$ ) binding are shown for each growth condition as well as the background due to the plastic ( $\square$ ). Total binding to matrix, pre-treated for 3 h. with 10 IU/dish of either heparinase or chondroitinase is represented respectively, by ( $\boxtimes$ ) and ( $\boxplus$ ). Non-specific binding has been estimated in the presence of a 500 fold excess of rLIF.

cell-free ECM preparation with heparinase and chondroitinase. Digestion of extracellular GAGs had no significant effect on the specific binding of  $^{125}\text{I}$ -LIF to UMR-derived ECM preparations (Fig. 2) indicating that the mechanism of LIF association with the ECM does not involve extracellular GAGs. Taken together, these findings demonstrate that exogenous LIF can form a specific complex with components of the ECM by means of a mechanism that does not involve the direct participation of carbohydrate side chains.

The molecular identity of the specific LIF binding sites expressed by UMR cells was further characterized by chemical cross-linking studies. UMR cell monolayers or cell-free ECM preparations were incubated under equilibrium binding conditions with  $^{125}\text{I}$ -LIF over a range of concentrations from 500 pM to 125 pM followed by cross-linking with both Sulpho-NHS and EDC. The results of the experiments are shown in Fig. 3. When  $^{125}\text{I}$ -LIF is cross-linked to cells grown in monolayer, two prominent and specific cross-linked species of 200 and 140 kD (after subtraction of 20 kD for the molecular mass of LIF) are detected. There is in addition a much fainter species of an approximate molecular mass of 350 kD apparent at high concentrations of  $^{125}\text{I}$ -LIF. Chemical cross-linking of  $^{125}\text{I}$ -LIF to cell-free ECM preparations, by contrast, reveals a single cross-linked species of 140 kD with only trace amounts of the 200-kD species detectable at the highest concentrations of  $^{125}\text{I}$ -LIF and no evidence for any larger species. This strongly suggests that the 200-kD species is cell-associated and the 140-kD species is, at least in part, extracellular and ECM-associated. Although these experiments do not permit precise quantification of the affinity of  $^{125}\text{I}$ -LIF for these species, a comparison of the intensity of cross-linked bands at different concentrations of LIF suggests that the 140-kD species is recognized with



**Figure 3.** Affinity cross-linking of <sup>125</sup>I-LIF to UMR-derived ECM and cells. Typical experiment. Similar results were obtained in two other experiments performed with UMR cells as well as with other cell lines (PYS and P19 cells).

somewhat lower affinity than the 200-kD species. These considerations suggest that the association of exogenous LIF with the ECM arises as a result of the formation of a specific complex with an ECM localized molecule of 140 kD and that

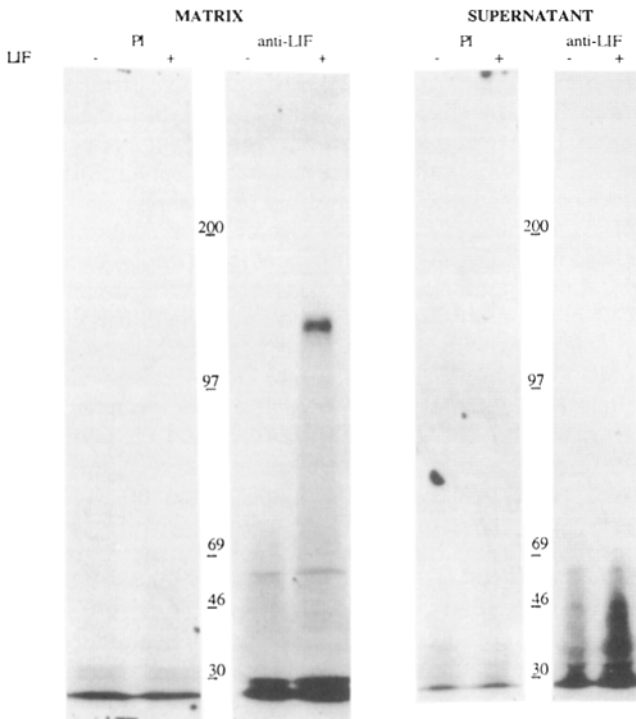
the high affinity sites ( $K_d = 50$  pM) detected by binding to cell monolayers results in part from LIF association with the 200 kD (and 350 kD) species.

#### Co-immunoprecipitation Analysis

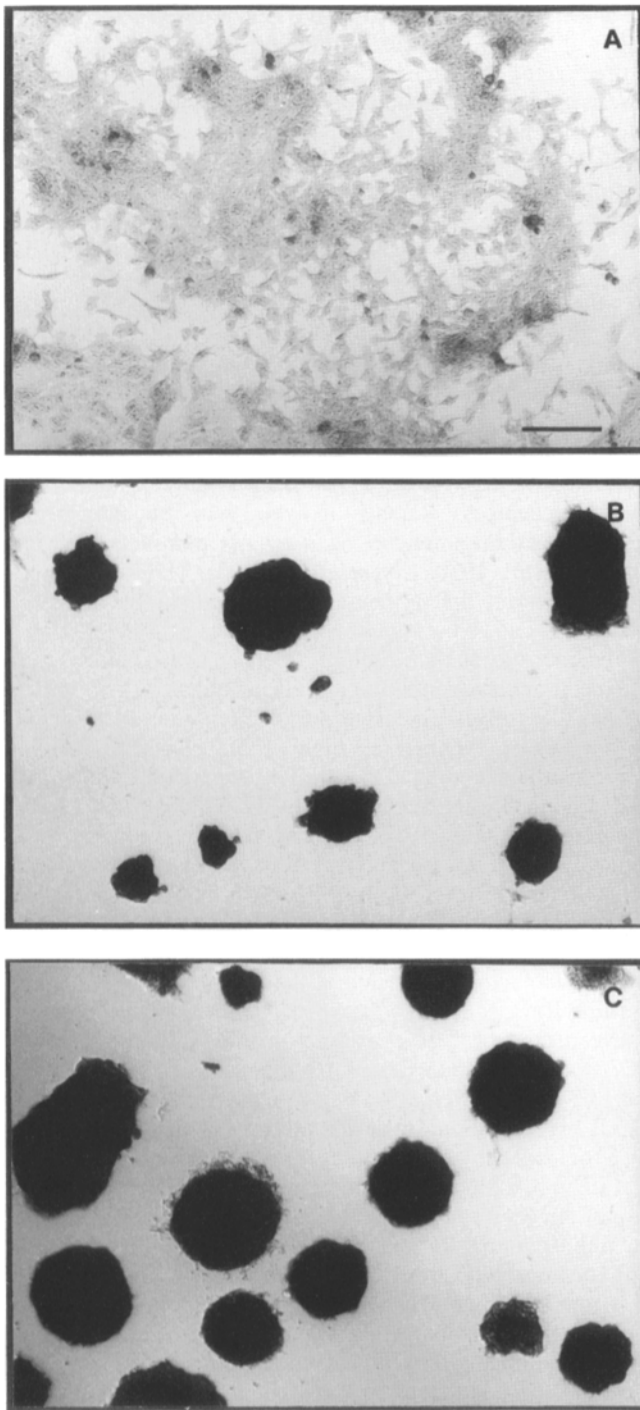
The above data indicates that radiolabeled exogenous LIF binds specifically to an ECM-localized species of 140 kD. As a complementary approach to this issue, we investigated the association of radiolabeled ECM components with unlabeled LIF using the ability of antibodies directed against mature LIF to co-precipitate LIF binding proteins. UMR cells were metabolically radiolabeled with [<sup>35</sup>S]methionine and a cell-free ECM prepared by removal of the cells with EDTA as before. The radiolabeled ECM extract was then incubated with 5 nM unlabeled LIF. This concentration was selected on the basis that it was about twofold higher than the  $K_d$  of the majority LIF binding sites detected by whole cell radioligand binding assay and equivalent to the concentration used in the analysis of LIF binding to cell-free ECM preparations shown in Fig. 2. The extracts were immunoprecipitated with anti-LIF antibodies immobilized on protein A-Sepharose and analyzed by SDS-PAGE and autoradiography (Fig. 4).

A single radioactive species was precipitated from the <sup>35</sup>S-labeled cell-free ECM extract with an apparent molecular mass of 140 kD. Immunoprecipitation of this species with anti-LIF antibodies was dependent upon previous co-incubation of the ECM extract with unlabeled LIF demonstrating that the 140-kD species was immunoprecipitated as a consequence of its affinity for soluble LIF. This species was not detected in culture supernatants subjected to the same procedures suggesting that this protein was predominantly localized in the cell-free ECM material associated with the tissue culture plate after removal of the cells.

The results of this experiment are therefore in accord with the results of chemical cross-linking studies in that they indicate that LIF can form a specific complex with a protein of 140 kD located in the ECM of UMR cells.



**Figure 4.** Co-immunoprecipitation of <sup>35</sup>S-labeled LIF-binding protein from UMR-derived cell-free ECM with anti-LIF antiserum. Immunoprecipitation with either anti-LIF antiserum or pre-immune serum (PI) of UMR cell supernatant and UMR-derived cell-free ECM extract in presence or absence of exogenous rLIF (5 nM).



**Figure 5.** Exogenous LIF bound to UMR-ECM prevents ES cell differentiation. ES cells ( $10^3$  cell/well) were cultured on UMR-ECM which was previously incubated for 3 h with (B) or without rLIF (10 ng/ml) (A) and then extensively washed with LIF-free medium. As a control, ES cells were grown to the same density in absence of ECM and in presence of rLIF (10 ng/ml) (C). After a 5-d incubation at 37°C in a humidified atmosphere, cultures were fixed and stained for alkaline phosphatase activity using the Sigma kit 86-R. Bar, 100  $\mu$ m.

### Biological Relevance

To determine whether the specific association of LIF to the 140-kD ECM-localized protein was biologically active, this

complex was tested for its ability to support growth of ES cells in an undifferentiated state (Smith et al., 1988). UMR matrix was pre-incubated in presence or in absence of exogenous LIF (to binding equilibrium), washed extensively and then used as a growth support for ES cells. These cultures were then stained for alkaline phosphatase (APase) activity, as a marker of undifferentiated ES cells (Bernstine et al., 1973). ES cells grown on pure UMR-ECM underwent differentiation as in LIF-free medium (Fig. 5 A). This result confirms that UMR cells do not produce matrix-associated LIF. In contrast, ES cells grown on UMR matrix that was pre-incubated with exogenous LIF, remained undifferentiated (Fig. 5 B) and grew in discrete colonies with typical ES morphology and APase expression in an equivalent manner to control cells cultured in the presence of soluble LIF (Fig. 5 C). Nonspecific interactions with matrix components or plastic cannot account for the retention of LIF since in control experiments performed with ECM-derived from either human or sparse UMR cells ( $10^4$  cells/cm<sup>2</sup>), ES underwent differentiation (data not shown). Hence, LIF retained in the ECM by its association with the 140-kD protein is able to invoke a biological response.

### Discussion

The experiments reported above demonstrate that the association of LIF with the ECM involves the formation of specific complex between LIF and an extracellular binding protein in the ECM since it is insensitive to heparinase and chondroitinase digestion. This binding protein can be identified by chemical cross-linking of <sup>125</sup>I-LIF to ECM localized sites or by co-precipitation by anti LIF antibodies and has a molecular weight of 140 kD. The chemical cross-linking experiments have also identified, in addition to an ECM localized binding protein, a LIF binding protein of 200 kD which is cell associated and significantly depleted from the culture dish by removal of the cells. Cross-linking to whole cells has also indicated the existence of an additional minor component of 350 kD. Moreover, soluble LIF retained specifically by the 140-kD protein located in the ECM is biologically active. Two important issues are therefore the molecular identity of these LIF binding proteins and their function in terms of LIF bioactivity.

Gearing et al. (1991) have reported the molecular cloning of a transmembrane protein which binds LIF with an affinity comparable to the "low affinity" class of LIF receptors reported here and in other studies (Hilton et al., 1988 and 1991; Godard et al., 1992). This molecule (hereafter termed LIF-R) has an apparent molecular mass of 190 kD and on the basis of its sequence relatedness to other receptors is a good candidate for a transmembrane LIF signal transducing receptor. Godard et al. (1992) have also identified, by chemical cross-linking of radiolabeled LIF to cells in suspension, a LIF receptor of  $\sim$ 250 kD. It would appear most likely therefore that the 200-kD species identified by chemical cross-linking experiments corresponds to the transmembrane form of the LIF receptor described by Gearing et al. (1991). Co-expression of the IL-6 receptor associated transmembrane protein gp130 with LIF-R has revealed an interaction between LIF-R and gp130 which results in the formation of "high affinity" binding sites of similar affinity to the high affinity ( $K_d = 50$  pM) class of binding sites observed in in-

tact cells in this and other studies. The minor cell associated 350-kD species detected in our cross-linking experiments might therefore represent a complex between the 200-kD LIF-R and gp130.

The ECM-localized LIF binding protein reported here differs from the transmembrane receptor LIF-R both in terms of molecular mass and its extracellular localization but interacts directly with LIF. In the course of isolating the transmembrane LIF-R, Gearing et al. (1991) isolated additional cDNA clones which encoded a truncated form of the LIF-R with a predicted molecular mass of 140 kD. This "secreted" form of the receptor (sLIF-R) yields a LIF-R species lacking the intracellular and transmembrane domains as well as one copy of the three type III fibronectin (FN III) repeat sequences of the extracellular domain. Recombinant sLIF-R was, in addition, found to remain physically associated with cells when expressed under the control of a heterologous promoter in COS cells (Gearing et al., 1991). An attractive hypothesis is therefore that the 140 kD ECM-localized species reported here is, in fact, the sLIF-R truncated form of the transmembrane LIF receptor.

Two other candidate proteins for the ECM-localized LIF binding protein exist. Layton et al. (1992) have reported the isolation of a 90-kD LIF binding protein from normal mouse serum based upon its ability to inhibit both the binding of radiolabeled LIF to target cells and its biological activity. The molecular mass of this protein would seem to be too low to represent any of the LIF binding proteins identified in this study. However, sequence analysis of this protein revealed that it was a truncated form of the LIF receptor lacking 48 residues at the amino terminus. If this molecule is derived from the secreted 140-kD sLIF-R it would seem probable that the protein is also truncated at the carboxy terminus, in the region of the FN III domains, to account for its reduced mass compared to the sLIF-R. This might indicate that the FN III domains may play some role in anchoring the sLIF-R (and therefore LIF) in the ECM and that the sLIF-R is subjected to further processing which controls its extracellular dissemination.

Under conditions which favored binding to the high affinity class of cell-associated LIF receptors, Godard et al. (1992) identified a species of 120 kD by cross-linking experiments. A similar protein has been determined to be a substrate for tyrosine phosphorylation induced in response to exposure of cells to LIF (Ip et al., 1992). In the latter case this molecule has been identified as gp130. It is unlikely, on two grounds, that the 140-kD species identified in this study and localized in the ECM is gp130. First, gp130 is a transmembrane protein and would be expected to be depleted when cells are removed from the culture dish. Second, there is strong evidence that LIF does not bind gp130 directly but rather gp130 binds to a complex of LIF and the LIF-R to form the high affinity class of binding sites (Gearing et al., 1992; Bruce et al., 1992). The ECM-localized 140-kD species appears to interact with LIF with lower affinity than cell associated binding sites and interacts directly with LIF. Our inability to detect the 120-kD species described by Godard et al. (1992) may well reflect the different cross-linking agents used in the two studies.

Taking these considerations together, therefore, we favor the hypothesis that the ECM localized LIF binding protein

is, in fact, the secreted form of the LIF-R described by Gearing et al. (1991). Conclusive proof of this possibility will require further experimentation, however, the hypothesis is attractive since it provides a biological function for this molecule; the control of delivery of LIF protein to target cells by sequestration of the factor in the ECM. These data not only provide a molecular mechanism for eliciting local action of LIF *in vivo* but also argue that ECM sequestration of LIF is intimately associated with the mechanics of LIF signal transduction.

Soluble forms of growth factor receptors have been reported in a number of cases including receptors which are related in structure to the LIF-R, the "hemopoietin receptors family." These include among others the receptors for IL-4, IL-5, IL-6, IL-7, and G-CSF (Mosley et al., 1989; Takaki et al., 1990; Taga et al., 1989; Goodwin et al., 1990; Fukunaga et al., 1990). It has been widely assumed that soluble receptors compete for ligand with transmembrane receptors and may therefore be antagonistic in function (Maliszewski et al., 1990; Layton et al., 1992). There are indications, however, that at least some ligand/soluble receptor complexes can be biologically active. Taga et al. (1989) have also demonstrated that a complex between IL-6 and its soluble receptor can elicit biological effects on target cells which do not themselves express the transmembrane form of the IL-6 receptor. Another example is the glycosyl-phosphatidylinositol-linked CNTF-R which is part of a functional and biologically active complex (Ip and Yancopoulos, 1992). It is also clear that LIF, in its ECM-associated form, is biologically active at least in terms of its ability to suppress ES cell differentiation. This would suggest that the association between LIF and the sLIF-R may represent a biologically active complex and that cells may be able to respond to LIF without requiring expression of the transmembrane form of the LIF-R.

Irrespective of these considerations, the data presented in this paper have demonstrated that the association of LIF with the ECM is an inherent property of the mature LIF protein and is brought about by an interaction between LIF and an ECM-localized binding protein. The studies have also shown that LIF is capable of associating with the ECM of cells that do not themselves express appreciable levels of M-LIF and is, in this context, biologically active. This implies that LIF can associate with the ECM if it has access to the ECM-localized binding protein. The role of the alternative amino termini may therefore be to regulate access to the ECM binding protein during biosynthesis of the nascent LIF protein. Equally the ECM localization of LIF requires the expression of the binding protein which can, in principle, be provided by a heterologous cell type. The phenomenon of ECM localization may extend beyond the expression of alternative LIF transcripts under *in vivo* conditions where heterologous cell types are in close physical proximity.

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